Bacterial Distribution and BETX Utilization in Subsurface Sediments from a Gasoline Spill

by Paula Krauter

Subsurface characterization of soil microorganisms has taken a prominent role in research efforts related to remediation of ground water contamination. The gasoline spill cleanup effort at LLNL affords an opportunity to study the collective effect of fuel hydrocarbons (FHCs) on the indigenous microbial populations. Presented here is a portion of a larger body of work that includes the analysis of 23 boreholes that characterizes the microbial ecology of the gasoline spill area. More information can be found in publications entitled "Effects of subsurface sediment characteristics on microorganism populations in a gasoline contaminated area" (UCRL-JC-115073) and "Changes in number and diversity of subsurface microbial communities after Dynamic Underground Stripping remediation" (UCRL-JC-115979, in progress).

Our focus here is to present an example of the number and activity of indigenous microorganisms and the environmental parameters that influence them. The study included assessment of nutrient and metals content, enumeration of indigenous microbial populations, evaluation of native bacteria capable of transforming FHCs, and assessment of common indigenous microorganisms ability to travel within the site's lithology.

Background

Uncontaminated sediments, collected 244 m southeast of the Gasoline Spill (GS), have a limited organic carbon (OC) content (0.02-0.07%) and yield very low numbers of viable bacteria ($\leq 10^3$). Within the GS, much larger OC concentrations (0.03-0.14%) are present and available as substrate for microbial growth. Aerobic bacteria and fungi capable of HC biodegradation generally initiate the oxidative utilization of such substrates by means of intracellular enzymes called oxygenases, which require molecular oxygen (Atlas, 1984). Since microorganisms require nitrogen and phosphorus for cell division, the availability of these additional nutrients is important for their growth. Therefore, tests were conducted to identify simple nutrients that would increase bacterial growth beyond that facilitated by the sediments. As a result, the amount of catalytically active cell mass and the availability of oxygen determine the overall rate of aerobic FHC biotransformation.

Soil lithology influences the ability of water, nutrients, and microorganisms to move through a soil column. An experiment was conducted to determine the time a culture of *Pseudomonas* bacteria migrated through six types of sediment found in the gasoline contaminated area.

Methods

Microbial samples were collected from borehole GSB-710, located 13.5 m north of the gasoline spill source, but well within the gasoline contaminated zone (Fig. 1). GSB-710 was drilled using a hollow stem auger. The core sediment samples were assayed for bacterial counts or for benzene oxidation within one day to one week after their removal from the field sampling

site. Those not assayed within one day were stored at 4°C. All dilutions, platings, and transfers of core-sediment material were performed in a laminar flow hood.

Aqueous suspensions of the sediment samples were prepared and serially diluted with sterile 0.1% sodium pyrophosphate. Aliquots of the resulting dilutions were spread-plated in triplicate on dilute 1% peptone, tryptone, yeast extract, glucose, and magnesium chloride agar medium (PTYG) with appropriate controls (Ghiorse and Balkwill, 1983). The resulting microbial colonies were scored with an automatic plate counter (New Brunswick, Biotrans III) or visually counted, and then reported as colony-forming units per gram of dry weight soil (CFU/gdw).

Total microorganism counts [acridine orange direct count (AODC)] were determined by fluorescence microscopy using a modified method of Hobbie *et al.* (1977). All stain and buffer solutions were pre-filtered though 25-mm, 0.2-um pore size membranes. The slides were examined under oil immersion at a magnification of 1000X with the use of a Zeiss Axioplan microscope equipped for epifluorescence measurement. The epifluorescence system included a mercury HB050 light source, a 451860 fluorescence illuminator, a 487910 blue exciter-barrier filter (450-490 SB) with an FT 510 chromatic beam splinter, and an LP 520 barrier filter.

Weighed samples of core-sediment material (100-300 mg) were assayed for their ability to catalyze [U^{14}- C]benzene conversion to [U^{14}-C]phenol, both in the absence and presence of 20 mM sodium formate, a potential electron donor for microbial reduced pyridine nucleotide regeneration (Taylor and Hanna, in progress).

Core material from borehole GSB-710 at the 19.5 m depth was used to study the effects of various nutrient supplements on the sediment sample populations of viable microorganisms. Identical 1:10 dilutions of sediment were made in sterile distilled water. The resulting slurries were transferred to autoclaved capped Erlenmeyer flasks. Nutrient additions were based on the concentration ranges that have been reported for these same agents in temperate surface mineral soils. Nutrient concentrations reported in Atlas and Bartha (1987) were as follows: organic matter, 0.40-10.0%; nitrogen, 0.02-0.05%; phosphorus, 0.01-0.2%; potassium, 0.17-3.3%; calcium, 0.07-3.6%; magnesium, 0.12-1.5%; and sulfur, 0.01-0.2%. Sediments from three depths were analyzed for mineral nutrient elements in the GS (Table 1). Sodium sulfate, potassium phosphate, hydrogen peroxide, urea, and distilled water each were added to the 1:10 diluted sediment suspensions. The flasks were incubated at room temperature for 2 days on a shaker table set at 140 rpm. Each of the samples was subsequently plated onto Bacto agar (n=10; controls n=15) at room temperature for 23 days.

Representative sediment samples were analyzed for Total Petroleum Hydrocarbons (TPH) using U.S. Environmental Protection Agency (EPA) procedures (EPA 5030, preparation; EPA 8015/8020, analytical). Metals and mineral nutrient elements were analyzed by appropriate EPA methods (310.1, 325.3, 353.2, 6010, 7196, 7471, or 9038). These analyses were conducted by certified contract laboratories. Samples were delivered to these laboratories on ice, usually just a few hours following their collection.

Table 1. Chemical analysis of three sediment cores from borehole GSB-710.

Analyte	Sediment sample concentration (mg/kg)			Detection limit (mg/kg)	Analysis method
	29.6 m	30.7 m	31.3 m		
Nitrate	<0.5	<0.5	<0.5	0.5	EPA 353.2
Potassium	1,100	650	930	3	EPA 6010
Chloride	40	10	40	10	EPA 325.3
Carbonate	<50	<50	<50	50	EPA 310.1
Bicarbonate	80	60	<50	50	EPA 310.1
Sulfate	<30	<30	40	30	EPA 9038
Iron	9,800	8,600	8,700	1	EPA 6010
Manganese	530	290	300	0.1	EPA 6010
Calcium	2,600	2,600	2,600	1	EPA 6010
Sodium	140	120	130	1	EPA 6010
Magnesium	3,000	2,400	2,600	1	EPA 6010

BETX utilization analysis was conducted in microcosms consisting of 250 mL screw-cap bottles and sealed with Mininert TM valves (Alltech cat. #95326). Bottles and caps were acid washed and sterilized before use. Microcosms contained 100 mL of solution in each bottle and 100 g of test sediment. A 1% yeast extract-tryptone-magnesium chloride media was used. No carbon source other than BTEX was supplied. Medium supplied the microorganisms with a nitrogen source and trace nutrients. Test 1 inoculm was 100 g of sediment and the native microorganisms adhering to the soil particles from borehole SVB-GP-014. A *P. aeruginosa* culture from borehole SVB-GP-014 (5.5 m from spill source) was used for test 2. Control microcosms were autoclaved 3 consecutive days and sodium azide was added to inhibit cell growth. The control series included chemical blanks and chemical and sterile soil blanks. The microcosms were spiked with 5 mg/L each of benzene, toluene, ethylbenzene and xylene for a total of 25 mg/L BETX. The gas chromatograph used was a HP GC 5890 equipped with a flame ionizing detector and a DB-1 and 4 capillary column. The temperature was 80 °C and the detection limit of the BETX compounds was 0.5 ug/L. Syringes were used to remove liquid samples from the microcosms to volatile organics apparatus (VOAs) using traditional methods. Rate of BETX utilization was calculated from the average of 4 replicate samples recorded as $mg/m^3/d$. The fifteenth day was chosen for test duration because the degradation half-life of benzene is 15 d (Howard et al., 1991).

Pseudomonas migration through sample sediment cores was studied by a simple test originally designed to study water movement at the Rocky Flats nuclear storage site. Sediments from borehole SIB-ETS-212 were used to study bacteria migrations in several types of sediment. Lithology was defined by the drilling geologist. These sediments were graded from coarse to fine grain sediments. The core samples were dried 24 hr at 55 °C to remove moisture. Core samples were extruded from the sample sleeves by a hydraulic press. The time water took to migrate bottom to top of the core was measured. Replicate cores were tested with a solution containing 1×10^9 CFU/gdw *P. fluorescens*. The cores were tested for colony forming units before and after the test runs.

Results

Both the colony-forming units (1% PTYG agar) and total (AODC) bacteria numbers were found to vary markedly throughout the borehole profile (Table 3). The highest concentration of sediment TPH, 300 mg/kg at 37.3 m, depressed the colony-forming bacteria (138 CFU/gdw), but did not seem to influence the total microorganism number (6.81×10^6 AODC/gdw). In fact, the presence of TPH and benzene did not significantly influence the total population numbers at any depth. Site-wide data did show a negative correlation of TPH and benzene to the population of heterotrophic bacteria. GS sediment sample viable plate counts did not correlate, positively or negatively, with TPH or benzene concentrations by the Pearson correlation test.

Oxidative benzene degraders were found in some of the GSB-710 sediment samples. However, their rates of benzene oxidation were not generally as high as those found in sediments from boreholes closer to the spill (Krauter et. al., 1994). The initial product being measured in this assay is [^{14}C-phenol] from [^{14}C-benzene]. Formate is used as an oxidizable cosubstrate. Results from Table 3 show that sediment samples with active benzene-oxidative bacteria (at depths of 5.3, 8.8, and 32.5 m) are not necessarily associated with the most highly populated areas (CFU/gdw and AODC/gdw values). Of the 16 sediment samples that were analyzed for benzene oxidation from GSB-710, 9 had measurable benzene oxidative activity. Of these 9 samples, only 2 were from sediments with relatively high CFU/gdw (25.8 and 30.6 m). Benzene oxidative activity also did not necessarily coincide with the occurrence of TPH (sediments from 5.3 and 8.8 m). However, due to the ongoing vapor extraction operations in the GS, any gasoline at these depths, may have been removed by the extraction process, leaving an oxygen-rich environment with low or undetectable TPH concentrations.

Greater percent trophic states exist in the sediments between 16.3 and 35.2 m than those above or below these depths. The percent trophic state is calculated by dividing the number of colony-forming units by total number of microorganisms. Percent trophic state indicates the percentage of the microorganisms that are metabolically active, whereas the remaining percentage of the population is assumed to be either dormant or could not grow on the given culture medium.

The increase in percent trophic values in the 16.3-to 35.2-m range may be due to the location of vapor extraction screens and the subsequent air circulation in this zone. The screens are located in 1.5-m sections at various depths, dependent on sediment grain size. Zone 3 is located at 16.2-17.4 m, zone 4 is located at 19.5-21.0 m, and zone 5 is located at 24.7-26.2 m. The vapor extraction process added approximately 10 to 14 mole % oxygen to the local area; a vapor sample from 17.2 m indicated mole % oxygen increased from 5.01 to 19.16; and vapor samples from 24.5 m indicated mole % oxygen increased from 0.56 to 11.04 (analysis by the Quantitative Mass Spectrometry Laboratory, LLNL).

Many enzymes require the addition of metal ions (Mg^{+2}, Mn^{+2}, Fe^{+2}, Zn^{+2}, etc.) in order to be activated. Magnesium, manganese, iron, and zinc were found in our sediment samples (Tables 1 and 2). The concentration of heavy metals found in the sediment samples (Table 2) were in the low range of concentrations found by Lindsay (1979) in uncontaminated soils.

Nutrient Enhancement

The growth of indigenous bacteria from GSB-710 at the 19.5 m depth increased from 9.15×10^3 to 3.64×10^4 CFU/gdw (4-fold) with the addition of 0.5 M NH_4Cl and to 4.46×10^4 (5-fold) with the addition of 0.02 M H_2O_2 (Fig. 1). All other nutrients were ineffective in increasing the viable plate counts.

Pseudomonas Migration in Different Sediments Types

Pseudomonas aurginosa was able to travel through silty sand, sandy silt, silt, and gravely silt 10 cm sediment core samples (Table 4). The organisms were unable to break through clayey silt, and silty clay test cores. The rate of travel of the cell culture through the sediment was 2.56 cm/min in silty sand. The migration rate decreased ten-fold in silt-based sediments (range of 0.28 to 0.44 cm/min). The presence of clay decreased the rate of travel by another ten-fold (range of 0.02 to 0.04 cm/min).

Table 2. Analyses of metals in subsurface sediments from borehole GSB-710.

	Sample concentrations (mg/kg)			Limit of detection (mg/kg)	EPA method	Background concentration[b] (mg/kg)
Analyte	29.7 m	30.7 m	31.3 m			
Chromium	11	10	12	1	6010	1–1,000
Copper	19	12	11	1	6010	2–100
Lead	8	6	7	1	6010	2–200
Mercury	<0.1	<0.1	<0.1	0.1	7471	0.01–0.3
Nickel	22	17	24	1	6010	5–500
Silver	<0.5	<0.5	<0.5	0.5	6010	0.01–5
Zinc	28	24	19	1	6010	10–300

[a]Analysis by Clayton Environmental Consultants, May 16, 1991.

[b]Ranges of concentrations of metals found in uncontaminated soils by Lindsay (1979).

BETX Utilization

A series of experiments was conducted in order to determine BTEX degradation rates and consumption preference of indigenous microorganisms. The decline of the BETX compounds in microcosm vessels was monitored over a 15 d period at room temperature (22 °C). The degradation rates of acclimated microorganisms varies species to species. In microcosms without sediment the utilization rate of ethylbenzene and p-xylene was greater than in microcosms with sediment (Table 5). The gasoline compounds are consumed in various preference orders perhaps related to compound availability in the liquid phase.

Table 3. Bacterial counts, benzene-oxidation rates, contaminant concentrations versus depth profile from borehole GSB-710.

Depth (m)	CFU/gram dry soil wt[a]	AODC/gram dry soil wt	Trophic state (%)[b]	[^{14}C]Phenol[c] formed		TPH[d] (mg/kg)	Benzene (mg/kg)
				With formate	Without formate		
1.3	2.65×10^4	6.39×10^7	0.04	0	0	0	0
3.1	8.3×10^3	8.28×10^6	0.01	---	--	--	--
5.3	1.30×10^3	3.63×10^6	0.10	1.4	0	0	0
8.8	2.59×10^2	9.39×10^7	0.04	1.3	1.8	0	0
12.5	$<1 \times 10^2$	8.41×10^5	0.01	0	0	0	0
16.3	1.88×10^4	4.67×10^5	4.03	0	0	0	0
18.4	7.75×10^4	1.20×10^6	6.46	0	0.4	--	--
21.7	3.53×10^4	5.73×10^5	6.16	1	1.6	0	0
23.4	1.48×10^4	6.54×10^5	2.26	0	0	0	0
25.8	8.33×10^5	9.48×10^5	87.9	1.4	0	0	0
28.5	1.97×10^2	2.61×10^6	0.01	0	0.1	--	--
29.7	1.53×10^2	6.65×10^5	0.02	0	0	0.4	0.17
30.6	3.97×10^5	1.02×10^6	38.92	1.4	0	0.8	0.058
32.5	6.15×10^3	9.97×10^5	0.62	1.2	1.0	0.3	0
35.2	3.96×10^5	2.81×10^6	14.1	0	0	0.8	0.14
37.3	1.38×10^2	6.81×10^6	0.002	0	0.7	300	3.00
41.3	4.88×10^2	3.82×10^5	0.13	0	0.7	0.3	0.005

[a]Average of triplicate spread plates in 1 % PTYG agar medium

[b]% trophic state is calculated value of [(CFU/gdw)/(AODC/gdw)] ×100

[c]Listed data represent nmol formed/15 min/100 mg dry soil wt

[d]TPH, total petroleum hydrocarbons

Table 4. *Pseudomonas* migration through sediment cores from the gasoline spill.

Grain	SIB-ETS-212 sediments	Rate of culture migration (cm/min)	Rate of water migration (cm/min)	Cell Count (cells/mL)
Coarse	silty sand	2.56	2.75	TNTC
	sandy silt	0.44	(2.14)	7.8×10^4
	silt	0.34	---	1.6×10^5
	gravely silt	0.28	(1.25)	1.1×10^4
	clayey silt	0.02	0.11	BDL
Fine	silty clay	0.04	0.40	BDL

Notes:

TNTC- too numerous to count

BDL-below detection level

--- test not run (limited number of sediment cores)

( ) test run on similar core

Table 5. Utilization rates of BTEX compounds of indigenous acclimated aerobic microorganisms at the LLNL gas spill analyzed by gas chromatography.

Compound	Test 1[a] Sediment culture $g/m^3/d$	Test 2[b] Solution culture $g/m^3/d$
Benzene	0.33	2.20 (3.10)
Toluene	O.24	2.70
Ethylbenzene	0.23	3.00
p-, m-xylene	0.21	3.00
o-xylene	<0.01	2.60

Notes:

[a]Indigenous microorganism population in sediment was approximately 1×10^5 CFU/gdw sediment, the dominant organism was *P. aergunosia*. Sediments obtained from SVB-GP-013, 45 feet depth.

[b]Utilized only test solution and media, a 1×10^7 population of P. aergunosia was added to each microcosm.

Rate of BETX utilization was calculated from the average of 4 replicate samples.

In test 1 the microorganisms degraded 0.33 $g/m^3/d$ of benzene. This moderate degradation rate may be due to sorption and vapor pressure of the contaminant rather than the meatbolic activity of the microorganism. BETX analysis from test systems provide data that the BETX compounds were consumed by the microorganisms in the sequence of decreasing vapor pressure of the compounds. The vapor pressure of BETX compounds are: benzene 12.7, toluene 3.8, ethlybenzene 1.3, and xylene 1.1, at 25°C. Additionally, the published $Kds_{(0.2\ organic\ C)}$ of benzene is 0.076, toluene 0.11, and xylene 0.25 (Seip et. al., 1986) provide data that the contaminant benzene does not tightly sorb onto sediment particles. The Kd of toluene from the gasoline spill site was typically 0.55 in clay/silty/sand sediment (site data table-Jennifer). The toluene utilization rate of 0.24 $g/m^3/d$ (Table 5) may be due, in part, to the sorption of toluene to the soil particles. In contrast, when BETX compound are introduced into liquid media containing the indigenous bacteria the degradation rate increases by almost ten times and the order of contaminant disappearance was ethylene and p-, m-xylene prior to utilization of benzene.

Discussion

This study was undertaken to determine the influence of several subsurface parameters and TPH content on the acclimated indigenous microorganisms in a gasoline contaminated plume. The viable bacterial populations existing within the contamination plume are those that have presumably survived the gasoline spill. In the capillary fringe area and the saturated zone, the numbers of viable (heterotrophic) aerobic bacteria were usually diminished. Several possibilities exist: the bacteria were exposed to toxic levels of TPH; the localized environment was anaerobic; or the routinely used 1% PTYG agar screening medium did not furnish the micronutrients for the growth of many bacteria that exist in this area.

The concentrations of heavy metals found in our sediment samples were below levels that could reduce soil enzyme activity (Doelman, 1981). However, under certain anaerobic conditions, the ionic forms of heavy metals may increase. The ionic forms of these metals, such

as mercury, silver, and copper, are lethal or inhibitory to microorganisms. Copper sulfate (2 ppm) in water has been used to prevent algal growth and silver oxide or metallic silver is bacteriostatic or bactericidal (Pelczar and Reid, 1972). In metabolically active areas of the gasoline plume the qxygen is depleted and the pH declines so metal concentrations may influence an additional factor that inhibits microorganisms.

All microorganisms require nitrogen for cellular growth. However, bacteria are diverse with respect to their nitrogen requirement. Some can fix atmospheric nitrogen; some use inorganic nitrogen; and others may require one or more organic nitrogen compounds. The GS had nitrate concentrations below detection limits in three samples from GSB-710 (Table 1). Among the nitrogen compounds tested thus far, ammonium chloride seemed to increase the sediment bacterial population the most under the experimental conditions employed (Fig. 1). The addition of peroxide also resulted in a large apparent increase in bacterial numbers.

Population profiles alone do not correlate with BETX utilization ability. Aromatic compounds found in gasoline such as benzene, toluene, ethylbenzene and xylene are confirmed or suspected carcinogens, even at low concentrations (Dean, 1985). Indigenous acclimated aerobic microorganisms at the LLNL gas spill utilize BETX compounds at various rates (Table 5). The rate of BTEX utilization could be increased by adding a pure culture of *P. aergunosia* to a microcosm without sediment. BETX compound sorption on soil particles and BETX volatility may effect the degradation rates by microorganisms. In Table 6 the increase of BETX degradation in the microcosms without sediment provide data suggesting that microorganisms consume BETX compounds more rapidly without the competing sorption effects of sediments such as clay. For example, the utilization of ethylbenzene increases from 0.23 to 3.00 $g/m^3/d$ when the combined effects of soil particles are removed, and cell population increased from 10^5 to 10^7 CFU/gdw.

The ranges of BETX utilization are within the ranges of in situ fuel hydrocarbon degradation reported by Bartha and Atlas (1987) of 0.001 up to 60 $g/m^3/d$. Soil microorganisms in optimized conditions may degrade fuel hydrocarbons at a rate of 65-800 mg/L/d in situ. The benzene degradation rate established by gas chromatographic analysis (0.33 $g/m^3/d$) was verified using ^{14}C labeled benzene (0.38 $g/m^3/d$). Indigenous organisms found in the heavily contaminated sediments were *Flavobactor spp., P. stutzeri, P. fluorscens, P. aeruginosa, and P. putida.*

Conclusions from the microbiological characterization include:

1. Microorganism population numbers vary with depth.
2. Indigenous microorganism communities population numbers do not relate to microorganism benzene degrading ability.
3. Pseudomonas spp. can migrate in sand and silty sediments. The microorganisms did not move through clayey silt and clay sediments.
4. The migration rate of water and cells through the sediment columns decrease with fine grain soils.
5. Indigenous microorganisms can utilize BETX. The microcosm without sediment had a higher BETX utilization rate suggesting that chemicals with weaker attachment to the particles are more readily degraded.

6. One hundred thirty microorganism cultures have been isolated and purified from TFF and are in long term storage. These cultures can be revived and studied in microcosms to evaluate BETX degradation ability. Approximately 40% are unknown species and may be new discoveries.

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